

REMARKS/ARGUMENTS

Claims 1-74 are pending in the application; claims 1-18, 31 and 32 are currently under examination; claims 19-30 and 33-74 have been withdrawn pursuant to a Restriction Requirement. Claims 1-3 and 7-11 have been amended. Support for the amendments to the claims is found throughout the specification as originally filed and, thus, no new matter has been introduced. New claims 75-77 have been added. Support for new claims 75-77 is found throughout the specification as originally filed and, thus, no new matter has been introduced.

The present amendment is submitted in accordance with the Revised Amendment Format as set forth in the Notice provided on the USPTO web site for the Office of Patent Legal Administration; Pre-OG Notices; dated 1/31/03.

Claims 1-18, 31 and 32 have been rejected under 35 U.S.C. §§ 101, 112, first paragraph, and 112, second paragraph. For the reasons set forth herein, each of the rejections is overcome.

The Invention

The present invention provides nucleic acids encoding a novel ABC family cholesterol transporter, SSG. The claimed sequences are useful for a number of purposes, including the diagnosis and treatment of cholesterol-associated disorders, including sitosterolemia, and for the identification of molecules that associate with and/or modulate the activity of SSG.

Objection

Claims 1-18, 31 and 32 have been objected to as reciting non-elected subject matter. In order to expedite prosecution, claims 1-3 and 8-11 have been amended to recite the elected subject matter of SEQ ID NO:3 and SEQ ID NO:4.

Rejection Under 35 U.S.C. § 101

Claims 1-18, 31 and 32 have been rejected under 35 U.S.C. § 101 as allegedly lacking utility. The Office Action alleges that the specification does not demonstrate the specific function of the protein of SEQ ID NO:3 or its relationship to any disease. The Office Action

alleges that the main utility of the nucleic acid and protein is to carry out further research to identify the biological function and possible diseases associated with the protein, and, therefore, it is not specific or substantial. In response, Applicants respectfully traverse the rejection.

As set forth in M.P.E.P. § 2107, "[a]n invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible."

Furthermore, to establish a *prima facie* showing that the asserted utility is not specific or substantial, "[t]he *prima facie* showing must contain the following elements: (i) An explanation that clearly sets forth the reasoning used in concluding that the asserted utility for the claimed invention is not both specific and substantial nor well-established; (ii) Support for factual finding relied upon in reaching this conclusion; and (iii) An evaluation of all relevant evidence of record, including utilities taught in the closest prior art. (See, M.P.E.P. § 2107II(C)(1)).

Applicants assert that *prima facie* showing of no specific and substantial credible utility has *not* been properly established, because (1) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention and details provided in the specification, and (2) the utility is specific, substantial and credible.

1. A Person Of Ordinary Skill In The Art Would Immediately Appreciate Why The Invention Is Useful

Applicants assert that a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention and the numerous details provided in the specification. As explained above, the present invention provides nucleic acids encoding Sitosterolemia Susceptibility Gene (SSG) polypeptides. SSG is a novel member of the ATP-binding cassette (ABC) family of transport molecules. Members of the ABC family use ATP to drive the transport of any of a large number of molecules across membranes. SSG is involved in the transport of cholesterol and other sterols, as well as other lipids across membranes, and is associated with the human disorder sitosterolemia. The SSG

polypeptides associate with heterologous ABC transporter proteins to form heterodimeric transporters that transport sterols (*e.g.*, cholesterol) and other lipids out of cells (*see, e.g.*, page 14, lines 24-27 of the specification).

As further evidence that the SSG polypeptides of the present invention are, in fact, ABC transporters that play a role in sitosterolemia, Applicants direct the Examiner's attention to the following two *SCIENCE* articles: (1) Allayee *et al.*, "An Absorbing Study of Cholesterol," *SCIENCE* 290:1709-1711 (1 December 2000), a copy of which is attached as Exhibit A; and (2) Berge *et al.*, "Accumulation of Dietary Cholesterol in Sitosterolemia Caused by Mutations in Adjacent ABC transporters," *SCIENCE* 290:1771-1775 (1 December 2000), a copy of which is attached as Exhibit B. The Berge *et al.* article demonstrates that the SSG polypeptides of the present invention, which are referred to therein (as well as in the present specification) as ABCG5 polypeptides, are ABC transporters. More particularly, the Berge *et al.* article demonstrates that ABCG5, *i.e.*, SSG, and ABCG8 normally cooperate to limit intestinal absorption and to promote biliary excretion of sterols and that mutated forms of these transporters predispose one to sterol accumulation and atherosclerosis (*see*, the Abstract). The Allayee *et al.* article comments on the importance of the Berge *et al.* work. More particularly, Allayee *et al.* acknowledge that the Berge *et al.* results address "a longstanding mystery in the lipid metabolism field: How is absorption of cholesterol regulated given that cholesterol appears to be passively taken up by intestinal cells?" (*see*, page 1709), and that Berge *et al.* provide "strong evidence that ABC transporter proteins pump plant sterols out of intestinal cells into the gut lumen, and out of liver cells into the bile duct" (*see*, page 1709). Allayee *et al.* further state that "although the transporters preferentially pump out plant sterols, they also appear to transport cholesterol out of cells because the absorption of cholesterol is dramatically increased in sitosterolemia" (*see*, pages 1709-1710).

Based on their role as ABC transporters, the specification teaches that SSG nucleic acids and polypeptides (as well as modulators, recombinant forms, or fragments of SSG) can be used to modulate sterol transport activity in cells, and are therefore useful in the treatment or prevention of any of a large number of sterol-associated, *e.g.*, cholesterol-associated, diseases

and conditions, including, but not limited to, sitosterolemia, familial hypocholesterolemia, hyperlipidemia, atherosclerosis, coronary heart disease, HDL deficiencies, gall stones, nutritional deficiencies, and other cardiovascular diseases (*see*, page 10, lines 7-12 of the specification). Upon reading the specification, one of skill in the art would immediately recognize the utility of SSG in the treatment of such sterol-associated diseases. For instance, an SSG polynucleotide or polypeptide introduced into a cell, *in vivo* or *ex vivo*, can be used to modulate the SSG activity in the cell (*see*, page 11, lines 1-3 of the specification).

Clearly, one of skill in the art would immediately appreciate that the ability to modulate SSG activity in a cell through the introduction of an SSG polynucleotide or polypeptide would be useful for the treatment or prevention of sterol-related disorders because SSG transporters associate with heterologous ABC transporter proteins to form heterodimeric transporters that act to transport cholesterol, other sterols, and other lipids out of cells. For example, sitosterolemia, an inherited lipid disorder, is associated with an increased absorption and decreased elimination of dietary cholesterol as well as plant sterols, such as sitosterol, which are normally not absorbed from the diet. With the recent identification of mutations in the *ABCG8* and *SSG* genes as the underlying cause of sitosterolemia, disruption of the active pumping back into the intestine of passively absorbed plant sterols and decreased hepatic secretion of the resultant high levels of these sterols are now believed to be the causes of this disorder. Clearly, modulating the transport of cholesterol, other sterols, as well as other lipids out of cells is beneficial to a patient having a sterol-related disorder such as sitosterolemia.

Furthermore, since it has been shown that mutation of the *SSG* gene predisposes one to sitosterolemia, one of skill in the art would immediately appreciate the utility of the *SSG* gene and SSG polypeptide in the treatment, prevention and diagnosis of this disorder. As such, one of skill in the art would immediately appreciate why the present invention is useful.

2. The Utility Is Specific, Substantial and Credible

The Office Action alleges that use of the nucleic acid and protein to carry out further research to identify the biological function and possible diseases associated with the protein does not define a "real world" use. Applicants respectfully point out to the Examiner that

the foregoing utilities are specific, substantial and credible. Again, based on their role as ABC transporters, the SSG nucleic acids and polypeptides of the present invention as well as modulators of SSG can be used to modulate sterol transport activity in cells, and can therefore be used in the treatment or prevention of any of a large number of sterol-associated, *e.g.*, cholesterol-associated, diseases and conditions, including, but not limited to, sitosterolemia, familial hypocholesterolemia, hyperlipidemia, atherosclerosis, coronary heart disease, HDL deficiencies, gall stones, nutritional deficiencies, and other cardiovascular diseases" (*see*, page 10, lines 7-12 of the specification).

Moreover, as explained in the specification, LXR activity increases the expression of SSG polypeptides. As such, SSG nucleic acids and polypeptides also provide useful markers for detecting LXR or RXR activity, *e.g.*, to screen for LXR or RXR agonists (*see*, page 11, lines 20-22 of the specification). SSG polypeptides can also be used to generate monoclonal and polyclonal antibodies useful for identifying cells involved in cholesterol transport (*see*, page 11, lines 23 and 24 of the specification).

Applicants assert that all of the foregoing utilities are specific, substantial and credible. Contrary to what is stated in the Office Action, these utilities do not simply encompass carrying out further research to identify the biological function and possible diseases associated with the protein. Instead, the SSG nucleic acids and polypeptides of the present invention have immediate specific, substantial and credible "real-world" uses. Therefore, Applicants respectfully request that the rejection under 35 U.S.C. § 101 be withdrawn.

Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 1-18, 31 and 32 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly nonenabled. To the extent the rejection is applicable to the amended set of claim, Applicants respectfully traverse the rejection.

As the Examiner is aware, claims 1-18, 31 and 32 are directed to nucleic acids encoding polypeptides for SSG, a novel member of the ABC family of transport molecules. Claim 1, as amended, recites an isolated nucleic acid encoding an SSG polypeptide, the SSG polypeptide comprising an amino acid sequence that is at least about 70% identical to an amino

acid sequence as set forth in SEQ ID NO:3, wherein the amino acid sequence comprises a signature sequence selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:6.

As explained above, SSG is involved in the transport of cholesterol and other sterols, as well as other lipids across membranes, and is associated with the human disorder sitosterolemia. The SSG polypeptide associates with heterologous ABC transporter proteins to form heterodimeric transporters that transport sterols (*e.g.*, cholesterol) and other lipids out of cells. Based on their role as ABC transporters, the SSG nucleic acids and polypeptides of the present invention as well as modulators of SSG can be used to modulate sterol transport activity in cells, and can therefore be used in the treatment or prevention of any of a large number of sterol-associated, *e.g.*, cholesterol-associated, diseases and conditions. Moreover, since it has been shown that mutation of the SSG gene predisposes one to sitosterolemia, one of skill in the art would immediately appreciate the utility of the SSG gene and SSG polypeptide in the treatment, prevention and diagnosis of this disorder. As such, the specification disclose functions/activities of the SSG polypeptides and, in turn, uses that flow from such functions/activities.

Moreover, the structure of the SSG polypeptides is clearly described in the specification. More particularly, the specification teaches:

[t]opologically, full-length SSG polypeptides include a "transport unit," an "ATP binding domain," "a hydrophobic domain," six "transmembrane regions," "motif A" or "P-loop," "motif B," and others.

These domains are well-known in the art and are characteristic domains of many of the ABC transporter family members. In addition, the specification provides exemplary amino acid sequences for both human and murine SSG polypeptides (*see*, SEQ ID NO:3 and SEQ ID NO:4) as well as polymorphic variants of such SSG polypeptides (*see*, page 12 of the specification). Moreover, the specification provides "signature" sequences for the SSG polypeptides (*see*, SEQ ID NO:5 and SEQ ID NO:6). In fact, claim 1 has been amended to recite that the SSG polypeptide comprises an amino acid sequence having a signature sequence selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:6.

As such, the specification provides both the function(s) and structure(s) of the SSG nucleic acids and polypeptides of the present invention. As such, one of skill in the art can practice the claimed invention *without* undue experimentation. Accordingly, Applicants urge the Examiner to withdraw the rejection under 35 U.S.C. § 112, first paragraph.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 1-18, 31 and 32 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Each of the Examiner's concerns and, in turn, Applicants' responses to those concerns are set forth below.

a. The Examiner has rejected claim 1, stating that the phrase "encoding an SSG polypeptide" is unclear.

In order to expedite prosecution, claim 1 has been amended to recite that the acronym SSG stands for Sitosterolemia Susceptibility Gene. As set forth in the specification and as explained above, the SSG polypeptide is a member of the ABC . In view of the amendment to claim 1 and the remarks regarding the SSG polypeptide set forth above in connection with the § 101 and § 112, first paragraph, rejections, the Examiner's concern is overcome. Accordingly, Applicants urge the Examiner to withdraw this portion of the § 112, second paragraph, rejection.

b. The Examiner has rejected claim 2, stating that the phrase "said polypeptide specifically binds to polyclonal antibodies" is unclear because the specific structure and amino acid sequence of the claimed polyclonal antibodies are not known or defined.

Applicants respectfully submit that one of skill in the art would clearly understand the metes and bounds of claim 2 and, thus, claim 2 is not unclear. Claim 2, as amended, recites: "[t]he nucleic acid of claim 1, wherein said polypeptide specifically binds to polyclonal antibodies generated against a polypeptide that comprises an amino acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:6." As such, claim 2 requires that the SSG polypeptide, which is encoded by the claimed isolated nucleic acid sequence, specifically binds polyclonal antibodies generated against a polypeptide that comprises an amino acid sequence of SEQ ID NO: 3, 5 or 6. Clearly, one of skill in the art knows how to make such polyclonal antibodies and how to determine whether such polyclonal antibodies

specifically bind a polypeptide. As such, the structure and function of the polyclonal antibodies recited in claim 2 are not unclear to one of skill in the art.

Moreover, it is pointed out that claims similar in both scope and format to pending claim 2 have repeatedly been allowed by the USPTO. In fact, a brief search on the USPTO website reveals that a countless number of claims similar in scope and format to pending claim 2 have issued in the United States. Accordingly, Applicants urge the Examiner to withdraw this portion of the § 112, second paragraph, rejection.

c. The Examiner has rejected claim 4, stating that the phrase "ABC polypeptide, and wherein said dimer exhibits sterol transport activity" is unclear.

As explained in the specification and above, the SSG polypeptide is a novel member of the ATP-binding cassette (ABC) family of transport molecules. Members of the ABC family use ATP to drive the transport of any of a large number of molecules across membranes. SSG is involved in the transport of cholesterol and other sterols, as well as other lipids across membranes, and is associated with the human disorder sitosterolemia. In one embodiment, the SSG polypeptide associates with heterologous ABC transporter proteins, such as ABC8 or ABCG8, to form a heterodimeric transporter that transports sterols (*e.g.*, cholesterol) and other lipids out of cells. As such, in view of the teachings in the specification, claim 4 is not vague and indefinite.

d. The Examiner has rejected claim 7, stating that the meaning of the acronym "ABC8" is unclear.

In order to expedite prosecution, claim 7 has been amended to recite that the acronym "ABC8" stands for ATP-Binding Cassette 8. In view of the amendment to claim 7, the Examiner's concern is overcome. Accordingly, Applicants urge the Examiner to withdraw this portion of the § 112, second paragraph, rejection.

e. The Examiner has rejected claim 8, stating that the phrase "moderately stringent hybridization conditions" is unclear.

In order to expedite prosecution, claim 8 has been amended to set forth exemplary "moderately stringent hybridization conditions." Support for this amendment to the claims can

be found, for example, on page 25 of the specification and, thus, no new matter has been introduced. In view of the amendment to claim 8, the Examiner's concern is overcome. Accordingly, Applicants urge the Examiner to withdraw this portion of the § 112, second paragraph, rejection.

f. The Examiner has rejected claim 9, stating that the phrase "stringent hybridization conditions" is unclear.

In order to expedite prosecution, claim 9 has been amended to set forth exemplary "stringent hybridization conditions." Support for this amendment to the claims can be found, for example, on page 25 of the specification and, thus, no new matter has been introduced. In view of the amendment to claim 9, the Examiner's concern is overcome. Accordingly, Applicants urge the Examiner to withdraw this portion of the § 112, second paragraph, rejection.

g. The Examiner has rejected claim 14, stating that the term "LXR agonist" is unclear because the structure and identity of the claimed "LXR agonist" is not known and recited in the claims.

As the Examiner is aware, the term "LXR agonist" is a term of art that is known to and used by those of skill in the art to refer to compounds that activate a class of nuclear-hormone transcription factors known as liver X receptors (LXRs). As explained in the specification, it has been discovered that enhancing LXR activity in cells, *e.g.*, intestinal, liver, kidney, macrophage or smooth muscle, *etc.*, can be used to increase the expression of SSG. As such, any compound known to activate LXR or to enhance LXR activity in cells can be used to increase in the expression of SSG. The specification provides examples of LXR agonists, but other LXR agonists can be used as well. For instance, the specification teaches that LXR agonists include, *e.g.*, cholesterol as well as synthetic compounds such as Compounds A, B, C, which are set forth in Figure 6 and others, which are described in, *e.g.*, U.S. Patent Application Serial Nos. 60/115,292, 60/124,525, 09/525861, and 09/479315. As such, "LXR agonist" is a term of art and its meaning is well known to those of skill in the art. In view of the art-recognized meaning of this term, the use of "LXR agonist" in claim 14 does not render it unclear

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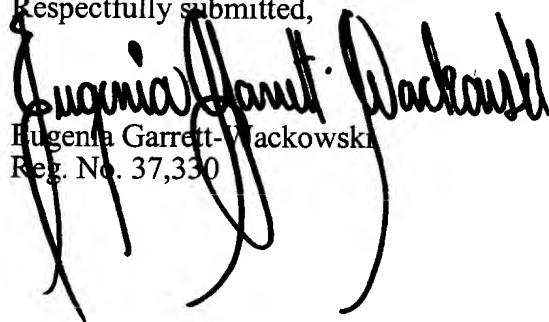
or indefinite. Accordingly, Applicants urge the Examiner to withdrawn this portion of the § 112, second paragraph, rejection.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,


Eugenia Garrett-Mackowski
Reg. No. 37,330

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
Attachments
EGW:lls
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from defects in the production of progenitor red blood cells, which are made after hematopoiesis shifts from the yolk sac to the liver at stage E12 of embryonic development. Expression of ADAR1 increases in the liver at this stage, suggesting that there is a critical transcript in liver tissue that requires efficient editing at this time. Apparently, the activities of ADAR1 and ADAR2 do not overlap sufficiently to enable one enzyme to take over the responsibilities of the other (defective) enzyme.

There are probably other transcripts that are edited by ADAR1, but chimeric mouse embryos do not survive long enough to allow other phenotypes to emerge. In their next set of experiments, Wang *et al.* induced teratomas (embryonic tumors) in nude mice by injecting them with either wild-type or ADAR1 heterozygous ES cells. The teratomas were composed principally of neural tissues in which the amount of editing of known transcripts could be measured. In stark contrast to heterozygous ADAR2 newborn mice that had no decrease in RNA editing (6), the teratomas formed in nude mice from heterozygous ADAR1 ES cells showed decreased editing of glutamate receptor (GluR-B R/G and GluR5 Q/R) and serotonin receptor transcripts (but no decrease in editing of the GluR-B Q/R site). Thus, ADAR1 editing is very dependent on whether two copies or only one copy of the gene are expressed. This sensitivity to gene dosage could arise if, for example, lower levels of ADAR1 result in splicing of the

transcript (that is, removal of noncoding introns) before editing, which would remove the essential ECS element.

In the heterozygous ES cells, a truncated protein (that has only the dsRNA binding domains) derived from the mutated copy of the *ADAR1* gene cannot be detected by Western blot analysis. Even if this truncated protein interfered with editing, this would not negate the possibility that ADAR1 is sensitive to gene dosage. However, clarification of the possible interference in the editing process by the truncated protein will have to await generation of ADAR1-deficient mice in which the entire gene has been deleted.

Fruit flies that completely lack any ADAR activity have been generated—this is much simpler to achieve in flies than in mice because flies have only one *ADAR* gene that is expressed exclusively in the CNS (7). The phenotype of the mutant flies shows parallels with that of the ADAR2 heterozygous mice. The fruit flies are viable and have a normal life-span, but they walk poorly, are unable to fly, and suffer progressive brain neurodegeneration. Editing is completely eliminated at known target sites in RNA transcripts that encode ion channels. The fly phenotype is consistent with the notion that RNA editing is important primarily in the fly CNS.

The generation of mice deficient in either of the two RNA-editing genes establishes beyond a doubt the importance of RNA editing. But what is the purpose of

this nuclear editing process? Does it “correct mistakes” in the genome, as appears to be the case for the Q/R site in GluR-B transcripts, or does it produce a diversity of protein products that do slightly different jobs? Only with the discovery of additional edited transcripts and testing of the functions of the edited and unedited forms will this question be answered. The evidence that editing is important and more widespread than previously thought seems particularly appropriate this year as we busily interpret the human genome sequence. The lesson that was learned from the mitochondrial and chloroplast genomes almost 20 years ago—that there is more to predicting the coding sequences of genes than simply identifying exons—should be remembered as we behold the final genome sequence of human nuclear DNA.

References

1. T. D. Fox, C. J. Leaver, *Cell* 26, 315 (1981).
2. P. S. Covello, M. W. Gray, *Nature* 341, 662 (1989); J. M. Gualberto *et al.*, *Nature* 341, 660 (1989).
3. R. Benne *et al.*, *Cell* 46, 819 (1986).
4. J. Scott, *Cell* 81, 833 (1995).
5. Q. Wang, J. Khillan, P. Gadue, K. Nishikura, *Science* 290, 1765 (2000).
6. M. Higuchi *et al.*, *Nature* 406, 78 (2000).
7. M. J. Palladino *et al.*, *Cell* 102, 437 (2000).
8. A. G. Polson *et al.*, *Biochemistry* 30, 11507 (1991).
9. C. Basilio *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 48, 613 (1962).
10. M. Higuchi *et al.*, *Cell* 75, 1361 (1993).
11. S. Maas, A. Rich, *Bioessays* 22, 790 (2000).
12. W. Keller *et al.*, *FEBS Lett.* 452, 71 (1999).
13. T. Melcher *et al.*, *J. Biol. Chem.* 271, 31795 (1996).
14. M. Paul, B. L. Bass, *EMBO J.* 17, 1120 (1998).
15. M. O'Connell, *Curr. Biol.* 7, R437 (1997).

PERSPECTIVES: BIOCHEMISTRY

An Absorbing Study of Cholesterol

Hooman Allayee, Bryan A. Laffitte, Aldons J. Lusis

Sterols are essential constituents of the membranes of animal and plant cells. Although structurally very similar, the sterols synthesized by animals and plants differ in the nature of their side chains; for example, the plant sterol sitosterol has the same ring structure as cholesterol (an important animal sterol) but differs in the side chain by an additional ethyl group (see the figure). Plant sterols taken in by animals in their food

cannot be used by mammalian cells and are not normally absorbed. The cellular machinery that allows selective absorption of animal sterols but not those of plants is defective in a rare, recessive disorder called sitosterolemia. Patients with this disease accumulate large amounts of plant sterols in most tissues, have elevated plasma cholesterol, and develop coronary heart disease at an early age (1, 2).

On page 1771 of this issue, Berge *et al.* (3) report the identification of mutations in two genes in sitosterolemia patients. The genes are new members of the ATP-binding cassette (ABC) family of transporters. Last year, another member of the gene family, *ABCA1*, was found to be mutated in Tangier disease. This disorder is characterized by defective efflux of

cholesterol from cells, which results in an inability to make high density lipoproteins (HDLs) (4). That finding proved to be a treasure trove for the field of lipid metabolism because it identified the transporter responsible for removing excess cholesterol from cells. The Berge *et al.* study seems likely to yield similar riches. In addition to clarifying how plant sterols are excluded from animal cells, their results address a longstanding mystery in the lipid metabolism field: How is absorption of cholesterol regulated given that cholesterol appears to be passively taken up by intestinal cells?

The intestine is a major barrier to the uptake of plant sterols: Less than 5% of dietary plant sterols are normally absorbed compared to 40% of the available cholesterol. Plant sterols also appear to be preferentially removed from the body by excretion into bile. Berge *et al.* provide strong evidence that ABC transporter proteins pump plant sterols out of intestinal cells into the gut lumen, and out of liver cells into the bile duct. Although the transporters preferentially pump out plant

H. Allayee and A. J. Lusis are in the Department of Medicine and the Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles (UCLA) School of Medicine, Los Angeles, CA 90095, USA. B. A. Laffitte is at the Howard Hughes Medical Institute, UCLA School of Medicine, Los Angeles, CA 90095, USA. E-mail: hallayee@ucla.edu; jlusis@mednet.ucla.edu



sterols, they also appear to transport cholesterol out of cells because the absorption of cholesterol is dramatically increased in sitosterolemia. Together with other recent studies, the following picture of sterol absorption emerges: Dietary sterols passively enter intestinal cells and a proportion of them are actively pumped back into the gut lumen by the ABC transporter proteins (see the figure). The control of cholesterol absorption is important for maintaining the correct levels of cholesterol in the blood and tissues and is achieved by modulating the expression of the ABC transporters.

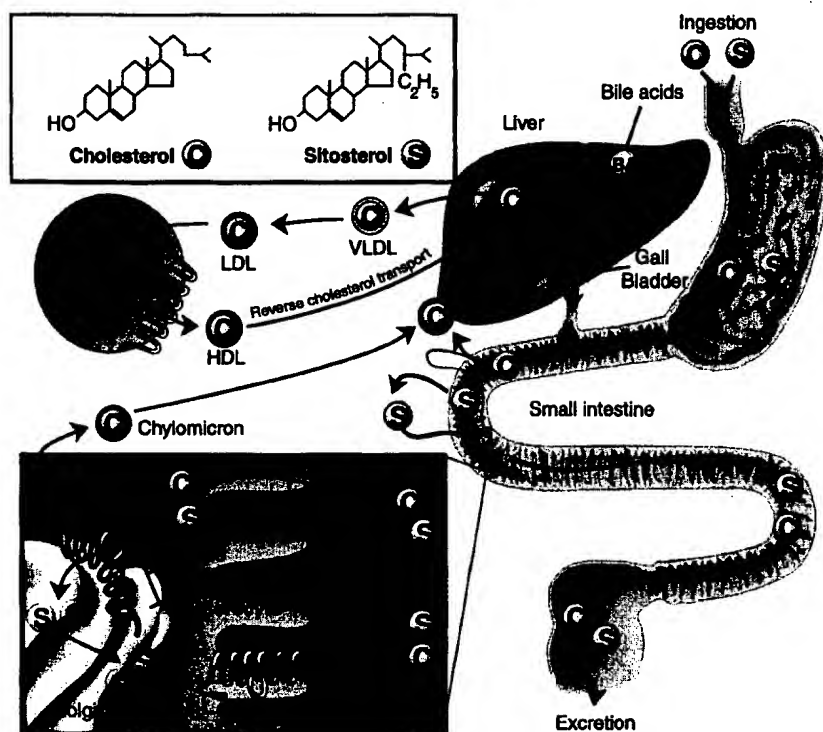
In a positional candidate gene approach, the authors used a combination of mapping information and functional data to identify the genes that are defective in sitosterolemia. Importantly, the sitosterolemia disease locus had been mapped to human chromosome 2p21 by Patel and colleagues 2 years ago (5). The authors also reasoned that the sitosterolemia genes might be regulated by the

liver X receptor (LXR) as this nuclear hormone receptor has been implicated in cholesterol homeostasis. Thus, the investigators used DNA microarrays to search for messenger RNA transcripts whose expression increased in response to a synthetic ligand for LXR. One such transcript encoded an ABC transporter (ABCG5), the gene for which mapped to chromosome 2p21. This gene happened to be adjacent to a second ABC transporter gene (ABCG8) that was switched on and off at the same time as ABCG5. Both genes were expressed in cells of the liver and intestine, and both were induced by a high-cholesterol diet fed to mice. Analysis of mutations provided strong evidence for the involvement of ABCG8 in four out of nine sitosterolemia families. The evidence that ABCG5 may be mutated in sitosterolemia patients is suggestive but not conclusive. The coordinated regulation of the two genes implies that the proteins they encode may unite to form an active transporter; thus, mutations in either gene could

give rise to sitosterolemia. Had ABCG5 and ABCG8 not been located so close together in the genome, their genetic mapping and subsequent identification could have been much more difficult.

Members of the ABC superfamily of transporters are integral membrane proteins that couple the energy derived from the hydrolysis of adenosine triphosphate to the transport of various substrates across cellular membranes. Active transporters consist of either a single polypeptide with two ABC domains and 12 transmembrane spanning helices (full transporter) or two polypeptides each with one ABC domain and six transmembrane spanning helices (half-transporters). Most half-transporters are located within intracellular membranes, whereas full transporters are usually found in the plasma membrane. Defective ABC transporters have been implicated in a variety of diseases including cystic fibrosis (ABCC7 transports chloride ions), adrenoleukodystrophy (ABCD1 transports very long chain fatty acids), and Tangier disease (ABCA1). Although ABCA1 is required for reverse cholesterol transport from peripheral tissues back to the liver for excretion, it may also be important in the regulation of intestinal cholesterol absorption (6, 7).

Nuclear receptors such as LXR are transcription factors that remain inactive in the absence of ligand. The binding of ligand to the receptor induces a conformational change that turns on the transcriptional activity of the receptor. The natural ligands for LXR are oxysterols, hydroxylated forms of cholesterol (8). The biological role of LXR is to detect high concentrations of cholesterol and to respond by increasing the expression of genes that limit its accumulation. In response to cholesterol, LXR (i) increases the synthesis of bile acids in the liver by inducing expression of cholesterol 7 α -hydroxylase (9), the rate-limiting enzyme of bile acid synthesis; (ii) increases cholesterol efflux from peripheral tissues (stimulating reverse cholesterol transport) by inducing ABCA1 and ABCG1 expression (7, 10); and (iii) inhibits cholesterol absorption in the intestine by activating ABCA1 and perhaps ABCG5 and ABCG8 (see the figure). The Berge *et al.* study suggests that ABCG5 and ABCG8 are regulated by LXR as their expression increased in response to an LXR ligand. However, final proof awaits the identification of the transcriptional regulatory sequences of these genes and examination of gene expression in mice deficient in LXR. The ability of LXR ligands to block cholesterol absorption should be examined in animals lacking either ABCG5 or ABCG8 to determine the relative importance of



Sterol metabolism in the body. The human diet includes sterols from both animal and plant sources, such as cholesterol (C) and sitosterol (S), respectively. (Bottom box) Ingested sterols enter gut epithelial cells (enterocytes) through an unknown route, perhaps by passive diffusion. The ABCG5 and ABCG8 proteins unite to form heterodimeric transporters in the plasma membrane and intracellular membranes (such as those of the Golgi) of enterocytes. These active transporters preferentially transport plant sterols (but also some cholesterol) out of enterocytes into the gut lumen, thereby decreasing sterol absorption. ABCA1 may participate in this process. Consequently, only a small percentage of the plant sterols that enter enterocytes are absorbed by the body. (Main figure) Absorbed sterols are packaged into chylomicrons for transport to the liver. In the liver, cholesterol and plant sterols may be (i) transported to peripheral tissues by lipoproteins (very low density lipoproteins, VLDLs; and low density lipoproteins, LDLs); (ii) converted to bile acids (BA); or (iii) transported out of the liver into the bile for excretion. In peripheral tissues, the ABCA1 transporter delivers cholesterol to high density lipoproteins (HDLs) for transport back to the liver.

these transporters in this process. The possible regulation of *ABCG5* and *ABCG8* (and other known target genes) by LXR suggests that LXR may be an excellent target for developing drugs to decrease serum cholesterol.

Although a full understanding of the regulation of cholesterol absorption in the intestine will require more work, the identification of genes mutated in sitosterolemia provides important insights into

this process. Four ABC proteins have now been implicated in the regulation of cholesterol homeostasis. Future studies will need to determine the structural and functional properties of these proteins and whether they act in concert or in separate pathways of cholesterol metabolism.

References

1. A. K. Bhattacharyya, W. E. Connor, *J. Clin. Invest.* 53, 1033 (1973).
2. I. Björkhem, K. M. Bobberg, in *The Metabolic Basis of*

Inherited Disease, C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, Eds. (McGraw-Hill, New York, ed. 7, 1995), pp. 2073–2099.

3. K. E. Berge *et al.*, *Science* 290, 1771 (2000).
4. Reviewed in J. F. Oram, A. M. Vaughan, *Curr. Opin. Lipidol.* 11, 253 (2000).
5. S. B. Patel *et al.*, *J. Clin. Invest.* 102, 1041 (1998).
6. J. McNish *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 97, 4245 (2000).
7. J. J. Repa *et al.*, *Science* 289, 1524 (2000).
8. D. J. Peet *et al.*, *Curr. Opin. Genet. Dev.* 8, 571 (1998).
9. D. J. Peet *et al.*, *Cell* 93, 693 (1998).
10. A. Venkateswaren *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 97, 12097 (2000).

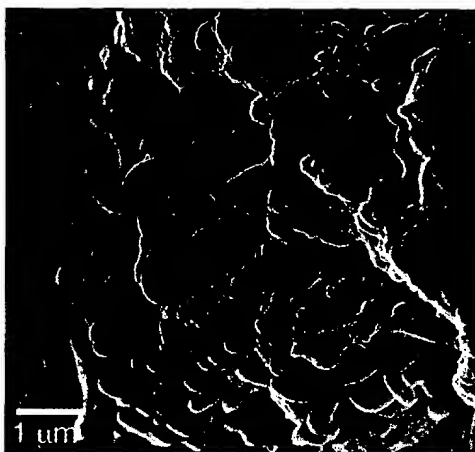
PERSPECTIVES: BIOGEOCHEMISTRY

Sulfate Reducers—Dominant Players in a Low-Oxygen World?

Crisogono Vasconcelos and Judith A. McKenzie

Sulfate-reducing bacteria may be one of the oldest forms of life on Earth. They can be traced back billions of years in the geologic rock record to the Early Archean (3900 to 2900 million years ago), when oxygen concentrations in Earth's atmosphere were low. Ancient sulfate-reducing bacteria left their first mark on their environment in pyrite minerals (FeS_2) as old as 3400 million years (1). Today, these microorganisms are widespread in marine and terrestrial aquatic environments. Their ability to adapt to extreme physical and chemical conditions enables them to play an important role in global geochemical cycles (2), but their role in the formation of ore deposits has remained controversial. Strong support for such a role is now provided by Labrenz *et al.* on page 1744 of this issue (3), who have discovered sulfate-reducing bacteria that can tolerate low levels of oxygen and can precipitate zinc sulfide minerals.

Throughout geologic history, the sulfur cycle was strongly correlated with the carbon cycle because the two cycles are intrinsically connected through microbial metabolism. The sulfur cycle thus constitutes one of the best examples of the impact exerted by living organisms on geochemical cycles (4). Dissimilatory sulfate-reducing bacteria use sulfate mainly as an electron acceptor (without assimilating sulfur) in the anaerobic oxidation of inorganic or organic substrates such as H_2 + CO_2 , lactate, acetate, and propionate. As a consequence of their metabolism, large amounts of reduced sulfide ions are produced and accumulated in their natural



The role of microbes in ore deposit formation. Scanning electron microscopy image of vibrio-shaped sulfate-reducing bacteria that are intimately associated with dolomite crystals produced in a culture experiment conducted at room temperature (9). The bacteria are 3 to 5 μm in length. The sample was prepared by chemical fixation and critical-point drying.

habitats. The sulfide ions combine with available metal ions to form insoluble products, most commonly FeS_2 , leading to the production and transformation of natural mineral deposits (5).

The importance of this major biogeochemical process is evident in the fluctuations in the sulfur isotope content of marine sulfate during the Phanerozoic, that is, during the past 570 million years. Bacterial sulfate reduction controls the isotopic composition of marine sulfate, driving the $^{34}\text{S}/^{32}\text{S}$ isotopic ratio, expressed as $\delta^{34}\text{S}$, to more positive values during periods with increased deposition of carbonaceous sediments. For example, in the early Phanerozoic, the $\delta^{34}\text{S}$ value of marine sulfate (as recorded in marine deposits) increases by about 15 per mil,

indicating a period with increased microbial sulfate reduction within anoxic (oxygen-deficient) sediments called black shales (6). Intervals with increased activity of sulfate-reducing bacteria, and associated increased impact on geochemical cycles, can thus be deciphered from the geologic record.

The role played by sulfate-reducing bacteria in natural processes is undoubtedly very important under anoxic or oxygen-free conditions. Anyone who has ever stepped into black stinky mud and smelled the H_2S released has experienced firsthand the activity of sulfate-reducing bacteria. These microbes undoubtedly play an important role in the early diagenetic alteration of sediments rich in organic matter. Their importance, however, for other geologic phenomena, such as the formation of sulfide ore deposits, remains controversial, not least because of their air intolerance. But this is not the case for the bacteria discovered by Labrenz *et al.* (3), which can tolerate low levels of oxygen (they are aerotolerant). These bacteria may be important players in geochemical cycling and in the concentration of metals into sediment-hosted sulfide ore deposits.

Using scuba divers to gain access to a flooded mine tunneled into a Pb-Zn ore deposit, Labrenz *et al.* were able to retrieve samples containing microbial biofilms. Applying microscale techniques, they demonstrate that the collected aerotolerant sulfate-reducing bacteria assemblage has the ability to form a pure precipitate of sphalerite (ZnS). The bacteria can scavenge zinc from waters with very low zinc concentrations (less than 1 part per million), essentially stripping the water of the metal. This observation has interesting implications for understanding how economic ZnS deposits may have formed. And it has even more exciting implications for possible biotechnological applications. Imagine if these aerotolerant sulfate-reducing bacteria could be used to remove trace metals, such as Zn, As, or Se, from contaminated drinking water! Because

The authors are at the Geological Institute, Eidgenössische Technische Hochschule-Zentrum, 8092 Zürich, Switzerland. E-mail: chris.vasconcelos@erdw.ethz.ch

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single immunization with collagen, with an extremely low incidence (<10%, Fig. 4A) (25). Immunizing CD200^{-/-} mice only once (26) resulted in disease onset as early as day 20 and a cumulative incidence of over 50% (Fig. 4A). That this result was not an artifact of gene targeting was illustrated by infecting C57BL/6^{+/+} mice with a replication-deficient adenovirus expressing a soluble Ig-fusion protein of CD200R (7, 27). Such mice were highly susceptible to CIA compared with mice receiving a control Ig-fusion protein construct (Fig. 4A). Both CD200^{-/-} and CD200R-Ig-treated animals developed moderate to severe arthritis (10) with synovial inflammation and formation of invasive pannus, resulting in cartilage and bone degradation seen normally only in CIA-susceptible animals (24) (Fig. 4B). Inflammatory cells in the arthritic joints were mainly CD11b⁺ cells (20), with a substantial proportion being CD68⁺ macrophages (10).

Because EAE and CIA are initiated by activation of self-reactive T lymphocytes (21, 25), enhanced disease could reflect hyperactivation of these cells in the absence of CD200. No evidence for T cell dysregulation in CD200-deficient environments was observed with a range of in vivo and in vitro experiments (10).

Thus, through CD200 expression, diverse tissues regulate macrophages, and probably also granulocytes, directly and continuously through interaction with the inhibitory CD200R (7). The consequences of loss of this pathway can be profound, rendering mice susceptible to tissue-specific autoimmunity and enabling accelerated reactivity of resident tissue macrophages, including those in the CNS. That these effects appear to be unrelated to T cell activation but rather the result of direct deregulation of effector pathways within the macrophage/myeloid lineage has important and broad implications for treatment of neurodegenerative diseases like Alzheimer's disease or for varied pathologies involving hyperactivation of the myeloid lineage.

References and Notes

- HLDA7: 7th Workshop and Conference on Human Leukocyte Differentiation Antigens, Harrogate, UK, 20 to 24 June 2000.
- A. N. Barclay, *Immunology* 42, 727 (1981).
- M. Webb, A. N. Barclay, *J. Neurochem.* 43, 1061 (1984).
- S. J. Davis et al., *Immunol. Rev.* 163, 217 (1998).
- F. Borriello et al., *Mamm. Genome* 9, 114 (1998).
- S. Preston et al., *Eur. J. Immunol.* 27, 1911 (1997).
- G. J. Wright et al., *Immunology* 13, 233 (2000).
- F. A. Lemckert, J. D. Sedgwick, H. Körner, *Nucleic Acids Res.* 25, 917 (1997).
- F. Köntgen et al., *Int. Immunol.* 5, 957 (1993).
- Supplementary data are available at Science Online at www.sciencemag.org/cgi/content/full/290/5497/1768/DC1.
- Single- and double-staining procedures used are detailed in (28). Primary antibodies were as in (12), and MOMA-1 (Bachem Bioscience, King of Prussia, PA), rabbit polyclonal anti-DAP12 antibody (15), and anti-CD45 (clone YTS165.1 obtained from S. P. Cobbold, University of Oxford, UK). Secondary antibodies were peroxidase-

or alkaline phosphatase-conjugated polyclonal anti-rat or anti-rabbit IgG (Jackson Immunochemicals, NJ). Hind limbs for histological analysis were prepared by means of a procedure adapted from (29).

- Single-cell suspensions of spleens were stained for mCD200 (13), B220, CD4, CD8, CD11b, Ly-6G, and F4/80 (PharMingen, San Diego, CA, and Caltag, Burlingame, CA) by standard procedures.
- OX90 was prepared by fusing splenocytes from rats immunized with a mouse CD200-rat CD4 fusion protein (6) with the Y3 myeloma, by standard procedures. The monoclonal antibody was selected by its capacity to bind recombinant mCD200-rat CD4 in an enzyme-linked immunosorbent assay (ELISA) and to bind those cells predicted to express CD200 (2, 3).
- G. Kraal, M. Janse, *Immunology* 58, 665 (1986).
- A. B. H. Bakker et al., *Immunol.* 13, 345 (2000).
- V. H. Perry, S. Gordon, *Trends Neurosci.* 11, 273 (1988).
- J. D. Sedgwick et al., *Proc. Natl. Acad. Sci. U.S.A.* 88, 7438 (1991).
- G. W. Kreuzberg, *Glia* 19, 312 (1996).
- M. B. Graeber, W. J. Streit, G. W. Kreutzberg, *J. Neurosci. Res.* 21, 18 (1988).
- R. M. Hoek et al., data not shown.
- C. S. Raine, *Lab. Invest.* 50, 608 (1984).
- S. R. McCall et al., *J. Immunol.* 161, 6421 (1998).
- EAE was induced and scored as described in (30) by subcutaneous immunization with 50 µg of myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide in complete Freund's adjuvant (CFA), but each mouse received 0.1 mg of H37RA *Mycobacterium tuberculosis* and intravenous injections of 100 ng of pertussis toxin on the day of immunization and 2 days later.
- I. K. Campbell et al., *Ann. Rheum. Dis.* 56, 364 (1997).
- W. C. Watson, A. S. Townes, *J. Exp. Med.* 162, 1878 (1985).
- CIA was induced by immunization intradermally with 100 µg of chicken type II collagen in CFA (0.25 mg H37RA *M. tuberculosis*) and scored only by clinical criteria as described in (24).
- The adapted mCD200R cDNA (residues 86 to 720) was subcloned in the Xho I site of a modified pCDM8Ig expression plasmid (31). The Hind III-Not I fragment of this was transferred to a modified pQ81-AdCMV5-GFP adenovirus transfer vector (Quantum Biotechnologies, Montreal, Canada), with an additional multicloning site 5'-AGATCTAAGCTTGCACGCGTATGCGGCGCATGGTACCATCTAGAGCGCAT-ATCGTTTAA AC-3' added between the Bgl II and Pme I sites. Recombinant adenovirus was produced with host QBI-293A human embryonic kidney cells (Quantum applications manual 24AL98). Virus expressing only the human Fc portion encoded by pCDM8Ig was used as control. Mice were infected 5 days before collagen immunization, and serum human Ig levels were subsequently monitored by ELISA.
- J. D. Sedgwick et al., *J. Exp. Med.* 177, 1145 (1993).
- R. Jonsson, A. Tarkowski, L. Klareskog, *J. Immunol. Methods* 88, 109 (1986).
- D. S. Riminton et al., *J. Exp. Med.* 187, 1517 (1998).
- E. E. Bates et al., *Mol. Immunol.* 35, 513 (1998).
- At various times after facial nerve transection (19), tissue was prepared for immunohistochemistry (11) of the brainstem area containing nucleus VII. To ensure valid comparison between different tissues, we made 20-µm coronal sections in a caudo-rostral direction starting in the cervical spinal cord, and advancing until the first appearance of nucleus VII. Eight-micrometer serial sections were prepared, and microglial activation was assessed (17, 19).
- We thank F. A. Lemckert and D. S. Riminton (Centenary Institute, Sydney, Australia) for technical assistance, N. Hutchings (University of Oxford, UK) for analysis of OX90 mAb, and L. Spargo (Hanson Centre, Adelaide, Australia) for advice on preparation of CIA tissue. We thank L. L. Lanier (UCSF, CA) and A. B. H. Bakker (DNAX, CA) for DAP12 antiserum, S. Gordon (University of Oxford, UK) for CD68 mAb, Y.-J. Liu (DNAX, CA) for support with DC studies, and M. Andonion for graphics assistance. Co-workers in Oxford were supported by the UK Medical Research Council. DNAX Research Institute is supported by Schering Plough Corporation.

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Accumulation of Dietary Cholesterol in Sitosterolemia Caused by Mutations in Adjacent ABC Transporters

Knut E. Berge,^{1*} Hui Tian,^{2*} Gregory A. Graf,¹ Liqing Yu,¹ Nick V. Grishin,² Joshua Schultz,³ Peter Kwiterovich,⁴ Bei Shan,³ Robert Barnes,¹ Helen H. Hobbs^{1†}

In healthy individuals, acute changes in cholesterol intake produce modest changes in plasma cholesterol levels. A striking exception occurs in sitosterolemia, an autosomal recessive disorder characterized by increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia, and premature coronary atherosclerosis. We identified seven different mutations in two adjacent, oppositely oriented genes that encode new members of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family (six mutations in *ABCG8* and one in *ABCG5*) in nine patients with sitosterolemia. The two genes are expressed at highest levels in liver and intestine and, in mice, cholesterol feeding up-regulates expressions of both genes. These data suggest that *ABCG5* and *ABCG8* normally cooperate to limit intestinal absorption and to promote biliary excretion of sterols, and that mutated forms of these transporters predispose to sterol accumulation and atherosclerosis.

In humans, the intestine presents a barrier that prevents the absorption of plant sterols and partially blocks the absorption of cholesterol.

This barrier is disrupted in the rare autosomal recessive disorder, sitosterolemia, which is characterized by hyperabsorption of plant ste-



rols such as sitosterol (1-3). Patients with sitosterolemia also hyperabsorb cholesterol and are usually hypercholesterolemic, which leads to the development of xanthomas (cholesterol deposits in skin and tendons) and premature coronary artery disease (2-5). Unlike individuals with other forms of hyperlipidemia, sitosterolemic subjects respond to restriction in dietary cholesterol and to bile acid resin treatment with dramatic reductions in plasma cholesterol levels (2, 3, 6).

Patients with sitosterolemia have markedly elevated (>30-fold) plasma levels of plant

sterols (sitosterol, stigmasterol, and campesterol) as well as other neutral sterols with modified side chains (1, 7, 8). Healthy individuals absorb only ~5% of the average 200 to 300 mg of plant sterols consumed each day (9). Almost all of the absorbed sitosterol is quickly secreted into the bile so that only trace amounts of sitosterol and other plant sterols remain in the blood (9). In contrast, subjects with sitosterolemia absorb between 15 and 60% of ingested sitosterol, and they excrete only a fraction into the bile (2-5). The liver secretes sitosterol into the bloodstream,

where it is transported as a constituent of low-density and high-density lipoprotein particles (1). With the exception of the brain, the relative proportion of sterol represented by sitosterol in tissues matches that in plasma (10 to 25%) (10). Hyperabsorption and inefficient secretion are not limited to plant sterols. Sitosterolemic subjects absorb a higher fraction of dietary cholesterol than normal subjects, and they secrete less cholesterol into the bile (2-5). Taken together, the genetic and metabolic data indicate that sitosterolemic patients lack a gene product that normally limits the absorption and accelerates the biliary excretion of sterols (2, 3).

The molecular mechanisms that limit sterol absorption are poorly understood, but clues have emerged recently from studies of the orphan nuclear hormone receptor LXR (11, 12). Mice treated with an LXR agonist show a marked decrease in cholesterol absorption and a corresponding increase in the intestinal expression of mRNA encoding the ATP-binding cassette protein-1 (ABCA1), a membrane-associated protein that has been implicated in the transport of cholesterol (11, 13). We hypothesized that sitosterolemic patients might have defects in other genes that limit cholesterol absorption and that the expression of these genes would be regulated by LXR. To test this idea, we used DNA microarrays to search for mRNAs that are induced by the LXR agonist T091317 in mouse liver and intestine (11, 14). A transcript corresponding to a murine EST (AA237916) was induced ~2.5-fold in the livers and intestines of treated mice. This EST resembled three *Drosophila* genes that encode ABC half-transporters (*brown*, *scarlet*, and *white*) expressed in the pigmented cells of the eye (15-17). These ABC half-transporters contain six membrane-spanning domains and form two types of heterodimers that transport guanine (*brown/white*) or tryptophan (*scarlet/white*). Because a human homolog of *white* (ABCG1) is implicated in cellular cholesterol efflux from macrophages (18, 19), we reasoned that the LXR-induced protein encoded by AA237916 might be involved in sterol trafficking in liver and intestine, and hence this gene was a candidate for the defect in sitosterolemia.

A full-length cDNA corresponding to

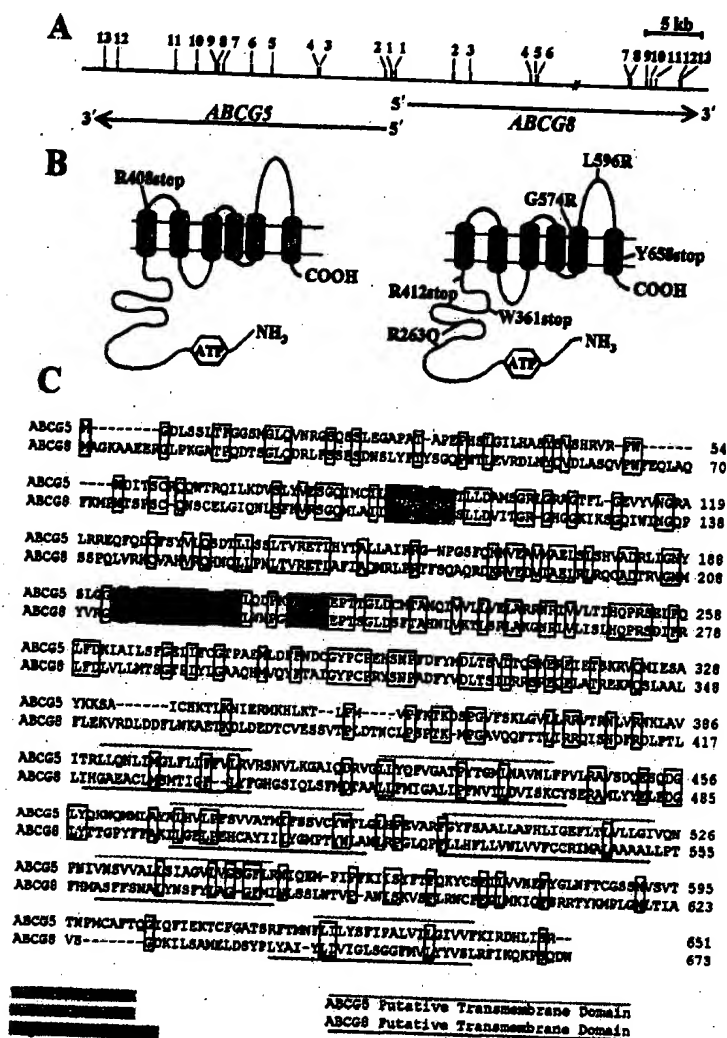


Fig. 1. Genomic structure (A), putative topology (B), and predicted amino acid sequences of ABCG5 and ABCG8 (C). ABCG5 and ABCG8 are located on chromosome 2p21 between markers D2S177 and D2S119. (A) ABCG5 and ABCG8 are tandemly arrayed in a head-to-head orientation separated by 374 base pairs. ABCG5 and ABCG8 are both encoded by 13 exons and each spans ~28 kb. (B) The mutations detected in patients with sitosterolemia (Table 1) are indicated on a schematic model of ABCG5 (left) and ABCG8 (right). (C) Predicted amino acid sequence of ABCG5 and ABCG8, which are 651 and 673 residues in length, respectively. Alignment of the inferred amino acid sequences indicates 28% sequence identity and 61% sequence similarity between ABCG5 and ABCG8. Both proteins are predicted to contain six transmembrane segments (22). The putative transmembrane segments of each protein are indicated by blue (ABCG5) or green (ABCG8) cylinders (B) and lines (C). The Walker A and Walker B motifs are highlighted in yellow and lavender, respectively. The ABC signature sequence (C motif) is indicated in purple.

¹Department of Molecular Genetics and McDermott Center for Human Growth and Development and

²Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390-9046, USA. ³Tularik Inc., Two Corporate Drive, South San Francisco, CA 94080, USA.

⁴Department of Pediatrics, Johns Hopkins University, Baltimore, MD 21205, USA.

*These authors contributed equally to the work.

†To whom correspondence should be addressed: Helen.Hobbs@UTSouthwestern.edu

AA237916 was isolated from a mouse liver cDNA library (OriGene Technologies, Rockville, Maryland), and this sequence was used to identify a human ortholog in the GenBank EST database (T86384). A full-length human sequence was obtained by iterative EST database searches and by cloning from human liver cDNA libraries (OriGene and Clontech, Palo Alto, California). The human cDNA predicts a 651-amino acid protein (Fig. 1C) that shares 80% sequence identity with the mouse sequence (20). Following the standard system of nomenclature in the ABC transporter field, this protein was named ABCG5. The amino-terminal half of ABCG5 contains the ATP-binding motifs (Walker A and B motifs) and an ABC-transporter signature motif (C motif), and the carboxyl-terminal region is predicted to contain six transmembrane (TM) segments (Fig. 1B) (17, 21, 22). A human EST clone (UniGene T93792) from ABCG5 mapped to chromosome 2p21 between markers D2S177 and D2S119, and the map position was confirmed using a radiation hybrid panel (23). Patel and colleagues previously mapped the human sitosterolemia gene to this same region of chromosome 2 in ten independent sitosterolemic families (24).

The structure of the human ABCG5 gene was characterized by analysis of a bacterial artificial chromosome (BAC) clone that contained the entire gene (Fig. 1A) (25). The gene spans ~28 kb and has 13 exons and 12 introns. The coding sequences and consensus splicing sequences were amplified from genomic DNA by polymerase chain reaction (PCR) and sequenced in nine unrelated subjects with sitosterolemia (Table 1). A sitosterolemic patient from Hong Kong (proband 9) was heterozygous for a transition mutation (CGA to TGA) in codon 408 that introduced a premature stop codon between TM1 and TM2. This mutation was not present in 65 normolipidemic individuals, including 50 Chinese subjects. No other potential disease-causing mutations were identified in ABCG5. A transversion in codon 604 that substituted a glutamic acid for glutamine (Q604E) in the loop between TM5 and TM6 was found in five sitosterolemic probands but was also present in 23% of the alleles from normolipidemic Caucasians ($n = 50$).

Genes encoding members of the ABC transporter family are often clustered in the genome (26). Because only a single ABCG5 mutation was identified in our collection of nine sitosterolemic probands, we searched the public and Celera genome sequences for other ABC transporters adjacent to ABCG5. An EST (T84531) that shared weak homology with the *Drosophila white* gene was identified and expanded using exons predicted by the computer program GENSCAN (27). Eleven of the 13 exons of the new gene, which we name ABCG8, were identified in

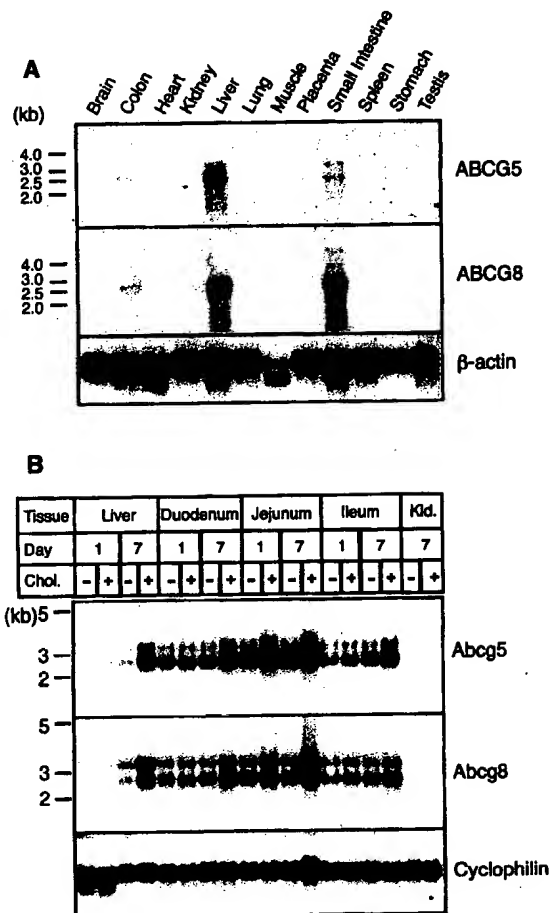
the databases, and the remaining two exons were identified by sequencing PCR-amplified cDNAs from human hepatic poly(A)⁺ mRNA. ABCG8 shares ~28% amino acid identity with ABCG5 (Fig. 1C). The ABCG8 sequence is most similar to ABCG1, which resembles the *Drosophila white* gene (16).

The translational start sites of ABCG5 and ABCG8 are separated by only 374 base pairs, and the two genes are arranged in a head-to-head orientation (Fig. 1A). Both genes contain a translation initiation codon with an upstream in-frame stop codon. The close proximity and opposite orientation of ABCG5 and ABCG8 suggest that the two genes have a bidirectional promoter and share common regulatory elements (28, 29). No obvious LXR response element was identified in the limited amount of sequence available at this time. Other gene pairs with bidirectional promoters form functional complexes (29), as may be the case for ABCG5 and ABCG8.

The predicted intron-exon boundaries of hu-

man ABCG8 were confirmed by DNA sequencing. The single-strand conformation polymorphism (SSCP) technique was used to screen the exons and flanking intron sequences of ABCG8 in the nine sitosterolemic subjects (Table 1) (30, 31). DNA sequencing of abnormally migrating fragments revealed six different mutations (Table 1 and Fig. 1B). The first patient to be described with sitosterolemia (proband 1) was homozygous for a nonsense mutation (1083G>A) in exon 7 (Fig. 1B) that introduced a premature termination signal codon at codon 361, terminating the protein before TM1 (1). Three other unrelated Caucasian sitosterolemic subjects (probands 3, 5, and 8) were heterozygous for the same mutation (6, 32). One of these probands (proband 5) was originally diagnosed with pseudohomozygous familial hypercholesterolemia (FH), an autosomal recessive disorder characterized by hypercholesterolemia, tendon xanthomas, and exquisite sensitivity to dietary cholesterol (6). Many of the patients originally diagnosed with pseudohomozygous FH were subsequently found to have sitos-

Fig. 2. Expression of ABCG5 (AF320293) and ABCG8 (AI320294) in human tissues (A) and the effect of cholesterol feeding on levels of ABCG5 and ABCG8 mRNAs in mouse liver and intestine (B). (A) Northern blot analysis of human tissues. The coding sequence of ABCG5 and ABCG8 were amplified from liver poly(A)⁺ RNA (Clontech), and the fragments were cloned into the plasmid vector pGEM-T (Promega, Madison, Wisconsin). The coding region of ABCG5 and the 3'-untranslated region of ABCG8 was amplified and the fragment radiolabeled (Megaprime DNA labeling System, Amersham, Uppsala) before incubation with the RNA blot (OriGene) in Rapid-hyb buffer (1×10^6 cpm/ml) (Amersham). The blot was washed and subjected to autoradiography for 18 hours using Kodak X-OMAT-blue film (Kodak, Rochester, New York) (40). (B) Cholesterol feeding induces coordinate increases in levels of *Abcg5* and *Abcg8* mRNA. Seven-week-old male mice (129S3/SvImj) were fed powdered rodent diet (Harlan Teklad, Madison, Wisconsin) in the absence or presence of cholesterol (2%, w/v). Mice were killed after 1 or 7 days in the light phase of a 12-hour dark-light cycle. Total RNA was isolated using RNA-Stat-60 (Tel-Test, Friendswood, Texas) from the liver and three equal segments of the small intestine (duodenum, jejunum, and ileum). The tissue RNAs were pooled from three animals, and aliquots (15 μ g) were used to make duplicate Northern blots (40). The mouse cDNAs for *Abcg5* and *Abcg8* were used as probes. Cyclophilin was used as an internal standard. The results were identical when probes generated from the 3' untranslated regions of both cDNAs were used.



terolemia, as was the case with this patient and proband 6 (6, 33). Proband 3 was heterozygous for another nonsense mutation in exon 13 that introduced a stop codon 15 residues from the carboxyl terminus of ABCG8. The resulting protein would lack part of TM6 and the short cytoplasmic domain, which contains a cluster of positively charged residues that may be important in positioning these proteins in the membrane (34).

Two missense mutations identified in ABCG8 produced nonconservative amino acid changes at positions that are conserved between the humans and mouse proteins, as well as in ABCG5. One Chinese patient (proband 4) was heterozygous for a missense mutation in exon 6 in codon 263 (Glu for Arg, R263Q). An Amish individual with sitosterolemia was homozygous for a missense mutation (Arg for Gly, G574R) in a residue that is conserved in mouse and human ABCG8. Genomic DNA was available from an additional three of the four living affected family members in this large Amish pedigree (35, 36), and these individuals were homozygous for this same missense mutation (20). A third nonconservative missense mutation was an arginine substitution for a leucine at codon 596. The corresponding sequence in ABCG5 is another nonpolar amino acid, glutamine. None of these three missense mutations were identified in 100 alleles from ethnically matched normolipidemic subjects, which is

consistent with their being disease-causing mutations. A common polymorphism (Cys for Tyr, Y54C) with an allele frequency of 23% in control subjects ($n = 100$ alleles) was also identified in ABCG8.

Thus, we identified two mutant alleles at the ABCG8 locus in four of the nine sitosterolemic patients. Four patients had a single mutant allele in ABCG8, and one patient had a single mutant allele in ABCG5. The identification of multiple different ABCG8 mutations in subjects with sitosterolemia, including nonsense mutations that appear incompatible with protein function, provides strong evidence that sitosterolemia is caused by defects in this gene. It also seems likely that the mutation we found in ABCG5 causes sitosterolemia, although the identification of additional mutations in this gene will be required to substantiate this hypothesis. It remains possible that mutations in another gene (perhaps a different ABC transporter) within the genomic interval mapped by Patel *et al.* (24) can cause sitosterolemia when present in combination with mutations in ABCG5 or ABCG8.

To determine whether ABCG5 and ABCG8 are regulated coordinately, we examined the tissue distributions of their mRNAs in humans and mice, and their responses to cholesterol feeding in mice. In humans, liver and the small intestine were the major sites of expression of both genes (Fig. 2A). For

both ABCG5 and ABCG8, one major transcript of 2.4 kb and 2.6 kb, respectively, but other transcripts were visible by RNA blotting. Additional studies will be required to determine the identity of these transcripts, which presumably result from alternative splicing or differential polyadenylation. In mice, *Abcg5* and *Abcg8* were expressed at higher levels in the intestine than in the liver, although the relative amounts of expression in these two tissues may be strain-specific. Inasmuch as the expression of these two genes is regulated by dietary sterols (see below), definitive studies of tissue expression in humans will require careful control of dietary intake.

If ABCG5 and ABCG8 protect against the accumulation of sterols, then their expression levels would be predicted to increase with cholesterol feeding. To test this hypothesis, we fed mice a high-cholesterol diet (2%), and they were killed after 1, 7, or 14 days. The levels of *Abcg5* and *Abcg8* mRNAs increased about twofold in intestine and over threefold in liver within 1 week of initiation of the high-cholesterol diet (Fig. 2B). These changes were maintained at 2 weeks (20). As expected, the plasma levels of cholesterol did not significantly change in the cholesterol-fed mice (from 95 mg/dl to 93 mg/dl), because mice rapidly and efficiently convert dietary cholesterol into bile acids and excrete both cholesterol and bile acids into the bile (37). LXR plays a central role in this regulated process by increasing the expression of multiple hepatic genes that promote bile acid synthesis and biliary secretion (12). The ligands for LXR include hydroxylated sterols that are derived from cholesterol (38, 39). Because ABCG5 is induced by an LXR agonist, it is possible that dietary sterols induce these genes through LXR.

In summary, our data suggest that ABCG5 and ABCG8 are ABC half-transporters that may partner to generate a functional protein. The juxtaposition of the corresponding genes on chromosome 2, the coordinate regulation of their mRNAs in the liver and intestine with cholesterol feeding, and the observation that mutations in either gene are associated with sitosterolemia suggest that these two proteins form a functional complex that mediates efflux of dietary cholesterol from the intestine, and thus protects humans from sterol overaccumulation. This protection is especially important in Western societies that consume high-cholesterol diets. Loss of function of these proteins causes sitosterolemia. Our results raise the possibility that subtle defects in these proteins or in their regulation may underlie the variable responses of healthy individuals to high-cholesterol diets.

Note added in proof: After submission of

Table 1. Molecular defects in nine unrelated individuals with sitosterolemia. Genomic DNA was extracted from cultured fibroblasts or lymphoblasts from the proband or another family member with sitosterolemia (37). All subjects had elevated plasma sitosterol levels (except proband 6 in which plasma sitosterol level was not measured). The age at the time of diagnosis or at the first appearance of xanthomas is provided (when available). The exons and flanking splice site consensus sequences were screened for sequence variations using SSCP and dideoxy-sequencing (31). None of the mutations were found in 100 alleles from normolipidemic controls. The nucleotides are numbered consecutively starting at the A of the initiation codon ATG. Plasma cholesterol levels were obtained from referring physicians or from publications. Abbreviations: ref., reference; yr, years; C, fasting plasma cholesterol level; chol, cholesterol; CAD, coronary artery disease; mo, months; LDL, low density lipoprotein; NI, not identified; amino acids: G, Gly; Q, Gln; L, Leu; P, Pro; R, Arg; T, Thr; W, Trp; Y, Tyr.

Patient (age)	Ethnicity	Mutant alleles	Nucleotide change	Amino acid change(s)	Comments	Ref.
1 (8 yr)	German/Swiss	ABCG8	1083G>A	W361Stop	Original case.	(1)
2 (13 yr)	Amish	ABCG8	1083G>A	W361Stop	C = 195 mg/dl	
3 (8 mo)	American	ABCG8	1720G>A	G574R	13-year-old died of CAD	(35)
4 (<10 yr)	Caucasian	ABCG8	1720G>A	G574R		
5 (5 yr)	American	ABCG8	1083G>A	W361Stop	C fell from 800 to 151 mg/dl on low-chol. diet	
6 (2 yr)	American	ABCG8	1974C>G	Y658Stop	C = 556 mg/dl	
7 (3.5 yr)	Chinese	ABCG8	788G>A	R263Q		
8 (2 yr)	Caucasian	NI	NI	NI		
9 (3.5 yr)	American	ABCG8	1083G>A	W361Stop	C fell from 375 to 201 mg/dl on low-chol. diet	(6)
10 (2 yr)	American	ABCG8	1234C>T	R412Stop	C fell from 753 to 106 mg/dl on low-chol. diet	(33)
11 (2 yr)	American	ABCG8	1787T>G	L596R		
12 (2 yr)	American	NI	NI	NI		
13 (2 yr)	Mexican-American	ABCG8	1234C>T	R412Stop	LDL-C fell from 380 to 200 mg/dl	
14 (3.5 yr)	Caucasian	NI	NI	NI		
15 (3.5 yr)	New Zealander	ABCG8	1083G>A	W361Stop	C fell from 718 to 127 mg/dl on low-chol. diet	(32)
16 (3.5 yr)	Chinese	ABCG5	1222C>T	R408 Stop	C = 620 mg/dl	
17 (<10 yr)	Chinese	NI	NI	NI		

the manuscript two additional mutations in *ABCG8* were identified by sequencing: (i) del547C resulting in a premature stop codon at amino acid 191 in proband 7 and (ii) P231T (691 A>C) in proband 4. No additional mutations were identified in *ABCG5*.

References and Notes

1. A. K. Bhattacharyya, W. E. Connor, *J. Clin. Invest.* **53**, 1033 (1974).
2. I. Bjorkhem, K. M. Boberg, in *The Metabolic and Molecular Bases of Inherited Disease*, C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, Eds. (McGraw-Hill, New York, ed. 7, 1995), vol. 2, chap. 65, p. 2073.
3. G. Salen et al., *J. Lipid Res.* **33**, 945 (1992).
4. T. A. Miettinen, *Eur. J. Clin. Invest.* **10**, 27 (1980).
5. D. Lütjohann, I. Bjorkhem, V. F. Bell, K. von Bergmann, *J. Lipid Res.* **36**, 1763 (1995).
6. I. Morganroth, R. I. Levy, A. E. McMahon, A. M. Gotto Jr., *J. Pediatr.* **85**, 639 (1974).
7. G. Salen et al., *J. Lipid Res.* **26**, 203 (1985).
8. R. E. Gregg, W. E. Connor, D. S. Lin, H. B. Brewer Jr., *J. Clin. Invest.* **77**, 1864 (1986).
9. G. Salen, E. H. Ahrens Jr., S. M. Grundy, *J. Clin. Invest.* **49**, 952 (1970).
10. G. Salen et al., *J. Lipid Res.* **26**, 1126 (1985).
11. J. J. Repa et al., *Science* **289**, 1524 (2000).
12. D. J. Peet et al., *Cell* **93**, 693 (1998).
13. R. M. Lawn et al., *J. Clin. Invest.* **104**, 25 (1999).
14. Total RNA was prepared from the liver, intestine, and kidney of C57BL/6mice treated with the LXR agonist T091317 (50 mg/kg). Duplicate RNA samples were labeled with two fluorescent dyes and hybridized to mouse GEM1 microarrays (performed at Incyte Genomics, Palo Alto, CA).
15. D. T. Sullivan, S. L. Grillo, R. J. Kito, *J. Exp. Zool.* **188**, 225 (1974).
16. P. M. Blingham, R. Lewis, G. M. Rubin, *Cell* **25**, 693 (1981).
17. C. Higgins, *Annu. Rev. Cell Biol.* **8**, 67 (1992).
18. J. Klucken et al., *Proc. Natl. Acad. Sci. U.S.A.* **97**, 817 (2000).
19. A. Venkateswaran et al., *J. Biol. Chem.* **275**, 147000 (2000).
20. K. E. Berge et al., unpublished observations.
21. J. E. Walker, M. Saraste, M. J. Runswick, N. J. Gay, *EMBO J.* **1**, 945 (1982).
22. D. T. Jones, W. R. Taylor, J. M. Thornton, *Biochemistry* **33**, 3038 (1994).
23. Chromosomal localization of *ABCG5* was confirmed by using primers derived from exon 7 of *ABCG5* to amplify a gene-specific fragment from the TNG panel of radiation hybrids from Stanford Human Genome Center (Research Genetics, Inc.). The result was submitted to the RH Server (<http://www.shgc.stanford.edu/RH/index.html>), which linked *ABCG5* to SHGC14952, which is between markers D2S177 and D2S119.
24. S. B. Patel et al., *J. Clin. Invest.* **102**, 1041 (1998).
25. The last three exons of *ABCG5* were contained in the GenBank sequence entry AC011242 and were further confirmed by PCR analysis from human genomic DNA. The remaining 10 exon/intron boundaries were determined by using PCR and cDNA primers to amplify the exon sequences and the intron/exon boundaries by using genomic DNA and cDNA primers followed by sequence analysis.
26. O. Le Saux et al., *Nature Genet.* **25**, 223 (2000).
27. The 3' end of *ABCG5* was located on BAC RP11-489K22, which had been partially sequenced, but no other ABC transporters were identified on this BAC. A BAC end sequence (BES) in the Genome Survey Sequence database that was located on BAC RP11-489K22 was used to search the Celera Human Fragments database. The public and Celera databases were used to assemble most of the genomic sequences in the region, resulting in the identification of EST T84531, which shared weak homology with the *Drosophila white* gene (16). The GENSCAN Web Server (<http://genes.mit.edu/GENSCAN.html>) was used to identify additional exons within this gene. The sequence of the ~30-kb region was assembled (excluding three gaps) using the Celera Human Fragments database and mouse ESTs in the public database.

28. S. A. Ikeda, A. Mochizuki, A. H. Sarker, S. Seki, *Biochem. Biophys. Res. Commun.* **273**, 1063 (2000).
29. R. Pollner, C. Schmidt, G. Fischer, K. Kuhn, E. Poschl, *FEBS Lett.* **405**, 31 (1997).
30. M. Orita, Y. Suzuki, T. Sekiya, K. Hayashi, *Genomics* **5**, 874 (1989).
31. H. H. Hobbs, M. S. Brown, J. L. Goldstein, *Hum. Mutat.* **1**, 445 (1992).
32. E. R. Nye, W. H. Sutherland, J. G. Mortimer, H. C. Stringer, *N. Z. Med. J.* **101**, 418 (1988).
33. P. S. Stell, D. L. Sprecher, *Top. Clin. Nutr.* **5**, 63 (1990).
34. G. D. Ewart, D. Cannell, G. B. Cox, A. J. Howells, *J. Biol. Chem.* **269**, 10370 (1994).
35. P. O. Kwitterovich Jr. et al., *Lancet* **1**, 466 (1981).
36. T. H. Beaty et al., *Am. J. Hum. Genet.* **38**, 492 (1981).
37. D. W. Russell, K. D. Setchell, *Biochemistry* **31**, 4737 (1992).
38. B. A. Janowski et al., *Nature* **383**, 728 (1996).

39. B. A. Janowski et al., *Proc. Natl. Acad. Sci. U.S.A.* **96**, 266 (1999).
40. V. Jokinen et al., *J. Biol. Chem.* **269**, 26411 (1994).
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From Marrow to Brain: Expression of Neuronal Phenotypes in Adult Mice

Timothy R. Brazelton, Fabio M. V. Rossi, Gilmor I. Keshet, Helen M. Blau*

After intravascular delivery of genetically marked adult mouse bone marrow into lethally irradiated normal adult hosts, donor-derived cells expressing neuronal proteins (neuronal phenotypes) developed in the central nervous system. Flow cytometry revealed a population of donor-derived cells in the brain with characteristics distinct from bone marrow. Confocal microscopy of individual cells showed that hundreds of marrow-derived cells in brain sections expressed gene products typical of neurons (NeuN, 200-kilodalton neurofilament, and class III β -tubulin) and were able to activate the transcription factor cAMP response element-binding protein (CREB). The generation of neuronal phenotypes in the adult brain 1 to 6 months after an adult bone marrow transplant demonstrates a remarkable plasticity of adult tissues with potential clinical applications.

Until recently, the fate of adult cells has been thought to be restricted to their tissues of origin. Cells are well known to be capable of replenishing damage in tissues in which they reside, such as blood, muscle, liver, and skin. However, the finding that adult cells could be reprogrammed to express genes typical of differentiated cell types representing all three lineages (mesoderm, endoderm, and ectoderm) when fused to cells in heterokaryons was quite unexpected (1–3). This degree of plasticity demonstrated that the differentiated state is reversible and requires continuous regulation to maintain the balance of factors present in a cell at any given time (4). The cloning of frogs (5) and later sheep (6) further showed that previously silent genes could be activated in adult nuclei. Although remarkable, these examples of plasticity all involved extensive experimental manipula-

tions. More recently, findings have been made that suggest that stem cells can assume diverse fates under physiologic conditions. Both transformed and primary neural cells can give rise to a range of phenotypes typical of their site of implantation within the central nervous system (CNS) (7, 8). Bone marrow cells can yield not only all cells of the blood but also cells with a liver phenotype (9). Perhaps the greatest plasticity yet demonstrated is the "homing" of bone marrow-derived cells to damaged muscle in irradiated dystrophic *mdx* mice (10, 11). Muscle-derived and CNS-derived stem cell-like populations have also been reported to reconstitute the blood and rescue lethally irradiated mice (11, 12). Here we report that after lethal irradiation, bone marrow-derived cells administered by intravascular injection yielded cells that expressed genes specific to neurons (neuronal phenotypes) in the CNS. Moreover, both the sources and the recipients of these cells were adults.

To examine whether bone marrow-derived cells could give rise to cells in the brain, adult marrow was harvested from transgenic

Department of Molecular Pharmacology, CCSR 4215, 269 Campus Drive, Stanford University, Stanford, CA 94305–5175, USA.

*To whom correspondence should be addressed. E-mail: hblau@stanford.edu